

Interim Progress Report for CDFA Agreement Number 14-0149-SA

Transgenic rootstock-mediated protection of grapevine scion by single and stacked DNA constructs

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INTRODUCTION

Collectively, a team of researchers (Lindow, Dandekar, and Gilchrist) identified, constructed and advanced to field evaluation five novel DNA constructs (Table 1) that, when engineered into grapevines, suppress symptoms of Pierce's Disease (PD) by reducing the titer of *Xylella fastidiosa* (*Xf*) in the plant, reducing its systemic spread in the plant, or blocking *Xf*'s ability to trigger PD symptoms. Current data indicate that each of the five transgenes dramatically reduces the disease levels under field conditions. The present field trial will be discontinued in July 2016 to be replaced with a second field trial evaluating the stacked gene transgenic rootstocks being developed by the current grant (CDFA 14-0149-SA) and completed via this proposal. The continuation of the basic research and the field trials to be described herein results from the field data indicating that several of the five DNA constructs, when incorporated into transgenic rootstock, have shown potential for protection of a non-transformed scion across a graft union. If successful, the obvious benefit would be that any unmodified (non-transgenic) varietal winegrape scion could be grafted to and be protected by transformed rootstock lines. This approach involves "stacking," a combination of distinct protective transgenes in a single rootstock line, which is intended to foster not only durability but also more robust protection of the non-transformed scion against PD. This approach to introduce pairs of these genes into adapted rootstocks was approved and

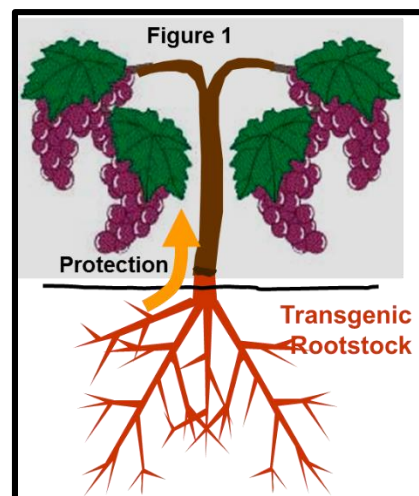


Figure 1, example scenario whereby a transgenic rootstock is being tested for ability to protect an untransformed scion from Pierce's Disease.

funded in 2014. Stacked transgene rootstock lines are now being received for greenhouse evaluation, grafting and evaluation first under controlled greenhouse conditions and then to produce ramets of the most suppressive transgenic-grafted lines for field evaluation beginning in 2017.

OBJECTIVES

1. Introduce pairs of protective constructs into an adapted grapevine rootstocks 1103 and 101-14.
2. Analyze each transgenic line to confirm correct insertion of the gene pairs and their expression in the respective rootstock
3. The resulting lines will be tested for efficacy by inoculation with *Xf* in a preliminary greenhouse experiment to identify the most protective lines from each combination of genes based on symptom expression, followed quantitative measurement of the presence and movement of the bacteria.

The primary motive for expressing genes in combination is to create durable resistance, resistance to *Xf* that will last the life of the vine. Since at least several of the five DNA constructs (Table 1) have biochemically distinct mechanisms of action, having two or more such distinctly acting DNA constructs “stacked” in the rootstock should drastically reduce the probability of *Xf* overcoming the resistance. With multiple, distinct transgenes, *Xf* would be required to evolve simultaneously multiple genetic changes in order to overcome the two distinct resistance mechanisms.

Additionally, there could be favorable synergistic protection when two or more resistance-mediating DNA constructs are employed. There are data indicating synergism in other crops. For example, the paper, “Field Evaluation of Transgenic Squash Containing Single or Multiple Virus Coat Protein Gene Constructs for Resistance to Cucumber Mosaic Virus, Watermelon Mosaic Virus 2, and Zucchini Yellow Mosaic Virus” (Tricoli et al 1995), describes the stacking of several genes for virus resistance in squash. Note, David Tricoli, the lead author in this paper, will be doing the stacking transformations in this proposal. Additionally, the Dandekar laboratory has successfully stacked two genes blocking two different pathways synergistically to suppress crown gall with (Escobar et al., 2001). Experiments proposed here will evaluate potential synergism in suppression of PD symptoms and in reducing *Xf* titer for inoculations distant from the graft union. Briefly, we describe information on the history and impact of the genes deployed as single transgenes currently in APHIS approved field trials. The subjects of this project are five specific DNA constructs (Table 1) that have shown to be effective in PD suppression under field conditions as single gene constructs and also appear to have potential in cross-graft-union protection described by the Lindow, Dandekar and Gilchrist in previous reports and are provided in the list of references.

Table 1. Genes selected to evaluate as dual genes in the 2nd generation field evaluation for suppression of Pierce's disease in grape

The table lists gene names, presumed function

<u>Gene</u>	<u>Function</u>
CAP	<i>Xf</i> clearing/antimicrobial
PR1	grape cell anti-death
rpfF	changing quorum sensing of <i>Xf</i> (DSF)
UT456	non-coding microRNA activates PR1 translation
PGIP	inhibits polygalacturonase/ suppressing <i>Xf</i> movement

CAP and PGIP: (Abhaya Dandekar)

The Dandekar lab has successfully participated in the two field plantings to investigate two greenhouse-tested strategies to control the movement and to improve clearance of *Xylella fastidiosa* (*Xf*), the xylem-limited, Gram-negative bacterium that is the causative agent of Pierce's disease in grapevine (Dandekar, 2013). A key virulence feature of *Xf* resides in its ability to digest pectin-rich pit pore membranes that connect adjoining xylem elements, enhancing long-distance movement and vector transmission. The first strategy tests the ability of a xylem-targeted polygalacturonase-inhibiting protein (PGIP) from pear to inhibit the *Xf* polygalacturonase activity necessary for long distance movement (Aguero et al., 2006). The second strategy enhances clearance of bacteria from *Xf*-infected xylem tissues by expressing a chimeric antimicrobial protein (CAP), that consist of a surface binding domain that is linked to a lytic domain the composition and activity of these two protein components have been described earlier (Dandekar et al., 2012).

rpfF, DSF (Steven Lindow)

The Lindow lab has shown that *Xylella fastidiosa* (*Xf*) uses diffusible signal factor (DSF) perception as a key trigger to change its behavior within plants (Lindow, 2013). Under most conditions DSF levels in plants are low since cells are found in relatively small clusters, and hence they do not express adhesins that would hinder their movement through the plant (but which are required for vector acquisition) but actively express extracellular enzymes and retractile pili needed for movement through the plant (Chatterjee et al. 2008). Accumulation of DSF in *Xf* cells, which presumably normally occurs as cells become numerous within xylem vessels, causes a change in many genes in the pathogen, but the overall effect is to suppress its virulence in plants by increasing its adhesiveness to plant surfaces and also suppressing the production of enzymes and genes needed for active movement through the plant.

PR1 and microRNA UT456 (David Gilchrist)

The Gilchrist lab is focused on the host response to *Xf* through identifying plant genes that block a critical aspect of grape susceptibility to *Xf*, namely the inappropriate activation of a genetically conserved process of programmed cell death (PCD) that is common to many, if not all, plant diseases in which cell death is the visible symptom of disease. Blocking PCD, either genetically or chemically, suppresses disease symptoms and bacterial pathogen growth in several plant-bacterial diseases (Richael et al., 2001, Lincoln et al. 2002, Harvey et al. 2007). In the current project with PD, a functional genetic screen identified novel anti-PCD genes from cDNA libraries of grape and tomato (Gilchrist and Lincoln 2011). Two of these grape sequences (PR1 and UT456), when expressed as transgenes in grape, suppressed Pierce's Disease (PD) symptoms and dramatically reduced bacterial titer in inoculated plants under greenhouse and field conditions. Assays with various chemical and bacterial inducers of PCD confirmed that the PR1 was capable of blocking PCD in transgenic plant cells (Sanchez et al., 2015a). It was then discovered that the mechanism blocking PR1 translation is due to the ability of the PR1's 3'UTR to bind to a region in the PR1 coding sequence to prevent translation. Sequence analysis of UT456 revealed a strong sequence complementarity to a region in the PR1 3'UTR that released the translational block of PR1 translation. Hence, the mechanism of suppression of PD symptoms depends on translation of either the transgenic or the endogenous PR1 message in the face of *Xf*-trigger cell stress (Sanchez et al., 2015b).

DESCRIPTION OF ACTIVITIES TO ACCOMPLISH OBJECTIVES

Construction of dual gene expression binaries:

The strategy is to prepare dual plasmid constructs bearing a combination of two of the protective genes on a single plasmid with single selectable marker. The binary backbone is based on pCambia1300 (Hajdukiewicz et al.; 1994). Binaries were constructed to express two genes from two 35S promoters (Figure 1). The DNA fragments containing transcription units for expression of the transgenes are flanked by rare cutting restriction sites for ligation into the backbone. The nt-PGIP used in these constructs is a modified version of the Labavitch PGIP that was modified in the Dandekar laboratory to include a signal peptide obtained from a grapevine xylem secreted protein (Aguero et al., 2006)

Binary plasmids capable of expressing two genes from the same TDNA (dual expressers) were constructed by Dr. Lincoln and are of the general form shown in Figure 2. All plasmids were transformed into *Agrobacterium* strain EHA105, the transformation strain for grape plant transgenics. As a check on stability of the dual expresser binary plasmid, the plasmid was isolated from two *Agrobacterium* colonies for each construct and the plasmid was used to transform *E. coli*. Six *E. coli* colonies from each *Agrobacterium* isolated plasmid (for a total of 12 for each construct) were analyzed by restriction digest to confirm that the plasmid in *Agrobacterium* is not rearranged. Table 2 shows when transformations were started by the UCD transformation facility. To ensure optimum recovery of the transgenic embryos, two versions of the plasmid with different antibiotic selectable markers were prepared. Hence, the dual inserts can now be subjected to two different selections that enables transformation to move forward in the fastest manner depending on which marker works best for each dual or each rootstock.

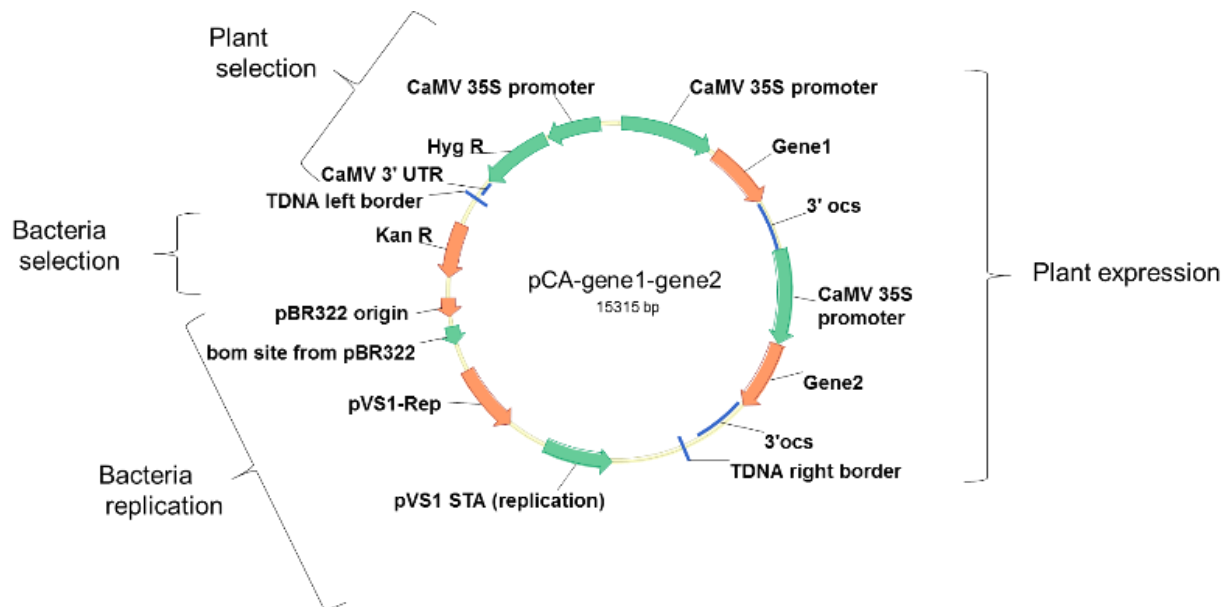


Figure 2. Dual expression binary expresses two genes within the same TDNA insert. This allows a single transformation event to generate plants that express two gene products.

Receipt of the transgenic rootstocks

Each dual protective gene plasmid will be introduced into embryogenic grapevine culture in a single transformation, i.e., conventional grapevine transformation in the Parsons Transformation Facility. The progress for each line is shown in Table 2.

Table 2. The current status of grape transformations into the rootstocks 1103 and 101-14.

Gilchrist March 2016 PD Transformation update																						
Genotype	Selection	PTF Code	PI	Start Date	Binary plasmid	Months in transformation																
						1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
1103	hygro	AT14119	Gilchrist	10/15/14	pCA-5fCAP-5oP14HT																	11
1103	hygro	AT14120	Gilchrist	10/15/14	pCA-5fCAP-5oUT456																	10
TS	hygro	AT14120	Gilchrist	4/29/15	pCA-5fCAP-5oUT456																	
1103	hygro	AT14121	Gilchrist	10/15/14	pCA-5PGIP-5oUT456																	
TS	hygro	AT14121	Gilchrist	4/29/15	pCA-5PGIP-5oUT456																	
1103	hygro	AT15007	Gilchrist	1/9/15	pCA-5PGIP-5oP14HT																	
1103	hygro	AT15008	Gilchrist	1/9/15	pCA-5PGIP-5FCAP																	
1103	hygro	AT15009	Gilchrist	1/9/15	pCA-5oP14HT-5oUT456																	
1103	hygro	AT15039	Gilchrist	5/26/15	pCA-5oP14HT-5orpff																	
1103	hygro	AT15040	Gilchrist	5/26/15	pCA-5oUT456-5orpff																	
1103	hygro	AT15041	Gilchrist	5/26/15	pCA-5fCAP-5orpff																	
1103	hygro	AT15042	Gilchrist	5/26/15	pCA-5PGIP-5orpff																	
1103	kan	AT15100	Gilchrist	8/28/15	pCK-rpff-P14LD																	
1103	kan	AT15101	Gilchrist	8/28/15	pCK-rpff-456																	
1103	kan	AT15102	Gilchrist	8/28/15	pCK-rpff-PGIP																	
1103	kan	AT15103	Gilchrist	8/28/15	pCK-rpff-CAP																	
TS	kan	AT15050	Gilchrist	4/17/15	pCB5o-UPUN																	

Key

	Embryos harvested, embryos to plants takes 3-9 months
	Plants rooted in vitro- Once plants are rooted in vitro, it takes 8 wks to transfer to soil and 2 wks to acclimate to soil
**	Plants delivered

The following images (Figure 3) illustrate the development of transgenic embryos, the initiation of roots and shoots from the transgenic embryo and, finally, the fully developed transgenic rootstock containing two of the transgenes. The quantitative analysis of the transgenic rootstocks has begun as illustrated in Figure 4. .



Figure 3. Rootstock 1103 embryos and developing plantlets with pCA-5fCAP-5oP14HT AT14119) transgenes inserted and the developed transgenic plant ready for RNA analysis and pathogenicity testing for response to *Xylella fastidiosa*.

Analysis of the transgenic rootstocks to confirm dual insertions

RNA from transgenic grape leaves is purified by a modification of a CTAB protocol and includes LiCl precipitation. The RNA is converted to cDNA by oligo dT priming and reverse transcriptase. PCR reactions are set up using the synthesized cDNA as template and specific pairs of primers designed against each of the 5 putative transgenes. The resulting products are separated by agarose gel electrophoresis (Figure 4). In this figure the bands revealed do correspond to two amplification targets in each transgenic plant. This technique, adapted by Dr. Lincoln to uniquely address this analysis, is referred to as multiplex analysis of each transgenic pair combination and allows for robust and rapid confirmation of the fidelity of the paired insertions.

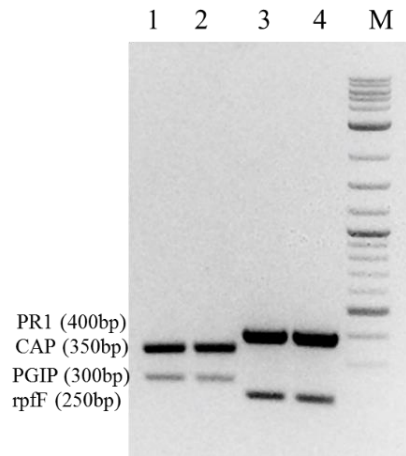


Figure 4. Leaf RNA analysis of 4 independent transgenic grape lines. Lanes 1 and 2 are putative CAP and PGIP dual expression lines. Lanes 3 and 4 are putative PR1 and rpff dual expression lines. Sizes of the expected products are shown.

Timeline for completion of delivery of the transgenic rootstock plants, the greenhouse and laboratory analysis, followed by the field planting the selected rootstocks grafted to the non-transgenic Chardonnay scions

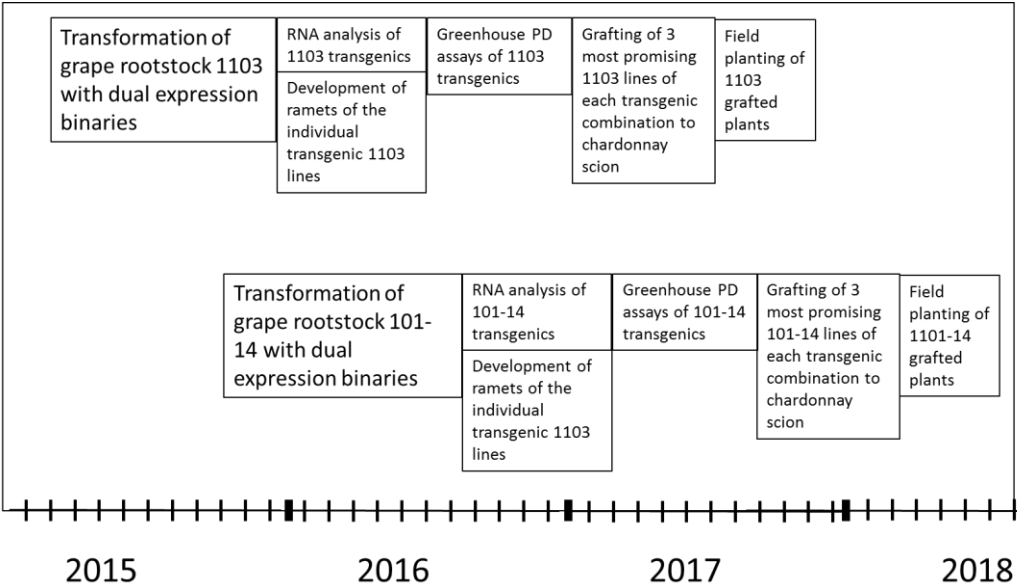


Figure 5. Anticipated Timeline for evaluation, propagation and planting of dual construct/susceptible scion combinations, fully transformed rootstock control, and untransformed susceptible control plants.

Our capacity to achieve all the objectives is essentially assured based on prior accomplishments. All techniques and resources are available in the lab and proven reliable, informative, and reproducible. This project will bring together a full time research commitment for this team of experienced scientists to Pierce's Disease. Each of the senior personnel, including Dr. Lincoln have been with this project since 2007 and have different skills and training that complement changing needs of this project in the areas of molecular biology, plant transformation and analysis of transgenic plants. This includes both greenhouse and field evaluation of protection against Pierce's Disease. Commercialization of the currently effective anti-PD containing vines and/or rootstocks could involve partnerships between the UC Foundation Plant Services, nurseries, and, potentially, with a private biotechnology company. The dual constructs have been assembled and forwarded to David Tricoli at the Parsons' Plant Transformation facility. The first transgenic plants have been delivered to Dr. Lincoln who has been begun the RNA the analysis to verify that each plant contains both of the intended constructs. The timeline shown in Figure 4 is on track.

RESEARCH RELEVANCE

Xylella fastidiosa (*Xf*) is the causative agent of Pierce's Disease (PD). Collectively, a team of researchers (Lindow, Dandekar, Labavitch/Powell and Gilchrist) has identified or constructed and advanced the evaluation of five (Table 1) novel genes (DNA constructs) that, when engineered into grapevines, suppress symptoms of PD by reducing the titer of *Xf* in the plant, reducing its systemic spread in the plant, or blocking *Xf*'s ability to trigger PD symptoms. These projects have moved from the proof-of-concept stage in the greenhouse to characterization of PD resistance under field conditions where current data indicate that each of the five transgenes effectively reduces the disease levels under field conditions. The current plants are mechanically inoculated, monitored for amount and movement of the bacteria, and for expression of typical PD symptoms. Symptom assessment is conducted by the PIs individually and by an independent team of evaluators lead by Deborah Golino. These existing field trials will continue through 2016. Importantly, each of the five DNA constructs, when incorporated into transgenic rootstock, has shown the ability to protect non-transformed scion, with obvious benefit that any of many unmodified varietal scions can be grafted to and be protected by any of a small number of transformed rootstock lines. Under Objective 1, the ability of transgenic rootstock to protect all or most of the scion, even at a distance from the graft union, will be tested. Objective 2 addresses the issue of durability, the capability of genetic resistance to avoid being overcome by evolving virulent versions of the *Xf* pathogen, a critical factor for a long-lived perennial crop such as grapevine. The approach under Objective 2 is "stacking," the combination of distinct protective transgenes in a single rootstock line, which is intended to foster not only durability but also more robust protection of the non-transformed scion against PD. The first of the stacked transgene rootstock lines are beginning to be released from transformation and are undergoing the first stage of RNA evaluation. It is anticipated that the first wave of transgenic rootstocks will be ready for field planting in 2017 and the final wave by 2018.

LAYPERSON SUMMARY

Xylella fastidiosa (*Xf*) is the causative agent of Pierce's Disease (PD). Collectively, a team of researchers (Lindow, Dandekar, Labavitch/Powell and Gilchrist) has identified or constructed and advanced the evaluation of five (Table 1) novel genes (DNA constructs) that, when engineered into grapevines, suppress symptoms of PD by reducing the titer of *Xf* in the plant, reducing its systemic spread in the plant, or blocking *Xf*'s ability to trigger PD symptoms. These projects have moved from the proof-of-concept stage in the greenhouse to characterization of PD resistance under field conditions where current data indicate that each of the five transgenes, introduced as single constructs, reduces the disease levels under field conditions. Importantly, preliminary data indicates

that each of the five DNA constructs, when incorporated into transgenic rootstock, has shown the ability to protect non-transformed scion, with obvious benefit: any of many unmodified varietal scions can be grafted to and be protected by any of a small number of transformed rootstock lines. The ability of transgenic rootstock to protect all or most of the scion, even at a distance from the graft union, is currently being tested. The objective described herein addresses the issue of durability, the capability of genetic resistance to avoid being overcome by evolving virulent versions of the *Xf* pathogen, a critical factor for a long-lived perennial crop such as grapevine. This approach involves “stacking,” a combination of distinct protective transgenes in a single rootstock line, which is intended to foster not only durability but also more robust protection of the non-transformed scion against PD. The stacking of genes is the next logical step toward achieving commercialization of transgenic resistance. Stacked transgene rootstock lines will be ready for evaluation in 2016 under controlled greenhouse conditions while ramets of the most suppressive transgenic lines are being produced for field testing; initiated by 2017. The proposed changes are the next logical step toward achieving commercialization of transgenic resistance.

PUBLICATIONS PRODUCED AND PENDING RELATED TO THIS PROJECT

- Sanchez, Juan, James Lincoln, and David Gilchrist, 2015a. Pathogenesis-related protein PR-1 interferes with programmed cell death and is synthesized under translational control (pending)
- Juan Sanchez, James Lincoln, and David Gilchrist, 2015b. The translation of pathogenesis related-PR-1 is triggered by a miRNA excised from grape coding sequences and the coding sequence of grape fan leaf virus. (pending)
- Lincoln, James and David Gilchrist. 2015. Pierce’s Disease suppression in grape by transgenic expression of DNA sequences capable of blocking programmed cell death. (pending)

FUNDING AGENCIES AND STATUS OF FUNDS:

Funding for this project is provided by the CDFA Pierce’s Disease and Glassy-winged Sharpshooter Board and the Regents of the University of California.

INTELLECTUAL PROPERTY. The grape plants containing the anti-PCD genes and the grafted rootstocks will require the use of several patented enabling technologies. Record of invention disclosures have been submitted to the UC Office of Technology Transfer. The research proposed reported herein will provide data on the activity and mechanism of action of the protective transgenes in grape relative to the presence, amount and movement of *Xylella fastidiosa* in the transformed and untransformed grape plants.

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